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Metabolically Engineered Micro-Organisms Having Reduced Production Of Undesired Metabolic Products

In the metabolic production of low-value added products like
5   ethanol, lactic acid, citric acid, amino acids and many
antibiotics, the yield of product on the substrate, i.e. the
amount of product formed per unit substrate consumed (often
given as kg product formed per kg substrate consumed), and
the productivity, i.e. the amount of product formed per unit
10   reaction volume and per unit time (often given as kg product
formed per m^3 reaction volume per hour) are the most
important design variables to optimise. In order to obtain a
high yield and productivity it is necessary to direct the
carbon fluxes from the substrate towards the metabolite of
15   interest at a high rate and at the same time minimize the
formation of all possible by-products. This often requires
engineering of the central carbon metabolism, which is
difficult due to the tight regulation in this part of the
cellular metabolism (Nielsen, 2001).
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Many fermentation processes have been optimised such that the
desired product is predominantly formed. However, due to the
complexity of cellular metabolism it is inevitable that by-
products are formed. This may be undesirable for at least
25   three reasons:

- The by-product(s) may be toxic to humans or animals.
- The by-product(s) may cause problems in the subsequent
  separation process or represent an environmental burden
- Formation of the by-product(s) results in a loss of
30   carbon, and the overall yield of product on the raw
  material is therefore below the theoretical maximum.

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The first reason is clearly problematic if humans or animals
may be exposed to the product, either directly or indirectly.
Typically, one chooses a cellular system that does not
produce toxins when products are made for human consumption,
5   e.g. fungal cells that do not produce aflatoxins are used for
production of food grade enzymes, and E. coli strains that do
not produce endotoxins are used for production of
pharmaceuticals. The second reason may especially be
problematic if the by-product has properties very similar to
10 those of the desired product, since separation of the active
product then requires very efficient separation principles,
which are often costly. In some cases the by-product may
also cause other types of problems, e.g. inactivation of the
desired product. Problems with loss of carbon in the by-
15 product is mainly a problem in connection with the production
of low-value added products like ethanol, citric acid, amino
acids and many antibiotics.

By way of example, a major problem in connection with ethanol
20 production by anaerobic fermentation of S. cerevisiae is a
substantial formation of glycerol as a by-product. Under
anaerobic growth conditions cytosolic NADH formed from
biomass formation can only be reconverted to NAD^+ via
glycerol formation (van Dijken and Scheffers, 1986). There
25 are two genes, GPD1 and GPD2, encoding glycerol-3-phosphate
dehydrogenase that regenerates NAD^+ from NADH while
converting dihydroxyacetone-phosphate to glycerol-3-
phosphate. Disruption of GPD2 results in some reduction of
glycerol formation, but the specific growth rate is also
30 significantly reduced (Valadi et al., 1998; Nissen et al.,
2000a). A double gpd1gpd2 deletion mutant strain is not able
to grow at anaerobic conditions, and introduction of a new

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pathway to regenerate NAD$^+$ was therefore attempted by
expressing a bacterial transhydrogenase (catalysing: NADH +
NADP$^+$ <=> NAD$^+$ + NADPH) of *Azotobacter vinelandii* in a double
gpd1gpd2 deletion mutant strain (Nissen et al., 2000a).
5   Expression of the transhydrogenase could, however, not
restore growth under anaerobic conditions. Enhanced
expression of a transhydrogenase is disclosed also in EP-A-
0733712.

10   WO99/46363 report on the expression of a phosphorylating
dehydrogenase resulting in a net transhydrogenase activity in
living cells with the aim to improve product formation.
Thus, through over-expression of *GDH2*, encoding a
phosphorylating NADH-dependent glutamate dehydrogenase (EC
15   1.2.1.12), they attempted to increase the ethanol formation
from xylose and glucose. Another attempt using the same
concept was to express a fungal NADPH dependent
glyceraldehyde dehydrogenase as described by Verho et al.
(2002). However, during growth on xylose the recombinant
20   cells also produced substantially more xylitol.

In another approach to improve ethanol production and
decrease glycerol formation Nissen *et al.* (2000b) engineered
the ammonia assimilation in *S. cerevisiae*. Through
25   disruption of *GDH1*, encoding NADPH-dependent glutamate
dehydrogenase, and over-expression of *GDH2* the production of
NADH in association with biomass synthesis was reduced
significantly resulting in a more than 40% reduction of the
glycerol yield (Nissen et al., 2000b). Furthermore, through
30   over-expression of the GS-GOGAT pathway for ammonia
assimilation, which is also NADH-dependent in *S. cerevisiae*,
both a reduction in the glycerol yield and an increase in the

ethanol yield was obtained (Nissen et al., 2000b). This
increase in ethanol yield is due to the additional
consumption of ATP in the GS-GOGAT pathway. This example
illustrates that redirection of the fluxes through the

5   central carbon metabolism can be obtained through engineering
other parts of the metabolism, particularly through
modulation of the redox metabolism, and this approach is
likely to function also for improving the production of other
products as the supply of NADPH for biosynthesis is often

10   limiting the capacity for production. While NADPH is almost
exclusively used as electron donor in biosynthesis in the
cell, NADH is primarily used for generation of free energy
(often in the form of high-energy phosphate-bonds in ATP).
Under some conditions, such as anaerobic growth of *S.*

15   *cerevisiae* on a fermentable sugar, surplus amounts of NADH
are formed which cannot by used in generation of ATP, and
this results in the formation of by-products, primarily
glycerol.

20   Valverde et al (1999)discloses an *E. coli* strain engineered
to express cDNA containing the *Pisum sativum GapN* gene which
encodes the non-phosphorylating glyceraldehyde-3-phosphate
dehydrogenase GAPN or GAPDHN (EC 1.2.1.9). The strain has
its native *Gap-2* gene encoding its NAD-dependent

25   phosphorylating glyceraldehyde-3-phosphate dehydrogenase
GAPDH disabled by an insertion. It is found that the
expression of GAPN re-establishes the ability of the strain
to grow aerobically on sugars, but the strain is still unable
to perform anaerobic fermentation.

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The present invention now provides a metabolically engineered
micro-organism having an operative first metabolic pathway in

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which a first metabolite is transformed into a second
metabolite in a reaction in which NAD is a cofactor for a
first enzyme, said reaction step producing NADH, and in which
said second metabolite is transformed into at least one
5   further metabolite in a reaction catalysed by a second
enzyme, and having an operative second metabolic pathway
characterised by an enzyme activity in excess of a native
level in respect of a third enzyme catalysing a non-
reversible reaction in which NADP is a cofactor and NADPH is
10   a product and in which said first metabolite is transformed
into a said further metabolite without the involvement of
said second enzyme.

In a micro-organism of the invention as described above, said
15   first metabolic pathway is preferably a native pathway.

In certain preferred embodiments, said first enzyme is a
phosphorylating dehydrogenase.

20   In certain preferred embodiments, including those referred to
immediately above, said second enzyme is a kinase.

In certain preferred embodiments, including those referred to
in the two paragraphs immediately above, said third enzyme is
25   a non-phosphorylating dehydrogenase, for instance said third
enzyme is GAPN (EC 1.2.1.9).

An example of a phosphorylating dehydrogenase first enzyme is
GAPDH (EC 1.2.1.12).
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In certain preferred embodiments, including all those
referred to above, at least one copy of a genetic sequence

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encoding said third enzyme has been recombinantly introduced
into said organism.

Preferably, a genetic sequence encoding said third enzyme is
operatively linked to an expression signal not natively
associated with said genetic sequence in said organism.

The micro-organism of the invention may preferably be a
yeast. This may be an ethanol producing fermenting yeast.
It may be a strain of Saccharomyces cerevisiae.

More generally, the micro-organism may be a species belonging
to the genus Saccharomyces, e.g. S. cerevisiae, S. kluyveri,
S. bayanus, S. exiguus, S. sevazzi, S. uvarum , a species
belonging to the genus Klyuveromyces, e.g. K. lactis K.
marxianus var. marxianus, K. thermotolerans, a species
belonging to the genus Candida, e.g. C. utilis C.
tropicalis, a species belonging to the genus Pichia, e.g. P.
stipidis, P. pastoris, P. sorbitophila, or other yeast
species, e.g. Debaromyces hansenii, Hansenula polymorpha,
Yarrowia lipolytica, Zygosaccharomyces rouxii or
Schizosaccharomyces pombe.

Concerning other micro-organisms (non-yeast), a non-
exhaustive list of suitable micro-organisms will include the
following:
Escherichia coli, Corynebacterium glutamicum, Aspergillus
niger, Aspergillus awamori, Aspergillus oryzae, Aspergillus
nidulans, Penicillium chrysogenum, Rhizopus oryzae.

The invention includes a genetically transformed micro-
organism containing one or more copies of an heterologous DNA

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sequence encoding GAPN operatively associated with an
expression signal and having a functional native or
heterologous expression capability for GAPDH (EC 1.2.12).

5   In a further aspect, the invention includes a method of
producing a desired metabolic product with decreased
production of an undesired metabolic product, comprising
culturing a micro-organism of the invention as described
above. The undesired metabolic product may be glycerol,
10 acetate or an amino acid, but it may also be any other
metabolite secreted by micro-organisms.

The desired product may be ethanol, lactic acid, citric acid,
an amino acid or an antibiotic.
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The invention will therefore be useful for the improvement of
production of ethanol but also of metabolites other than
ethanol in micro-organisms. Thus, Porro et al. (1999)
describe production of lactic acid in yeast through deletion
20 of pyruvate decarboxylase activity and expression of a
heterologous activity of lactate dehydrogenase. In the
conversion of pyruvate to lactate there is regeneration of
NAD^+ as is the case in the overall conversion of pyruvate to
ethanol. The overall conversion of a sugar to lactic acid
25 therefore has high similarity with the conversion of a sugar
to ethanol, and the invention will consequently have a
positive effect on lactic acid production.

In the production of citric acid by the filamentous fungus
30 Aspergillus niger there is a net formation of NADH in the
conversion of sugar to citric acid, i.e. NADH is generated at
the location of glyceraldehydes-3-P dehydrogenase and

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pyruvate dehydrogenase. Through expression of GAPN it will be
possible to replace part of the net formation of NADH with a
net formation of NADPH, which is needed for protein
synthesis. Hence, sugar that may otherwise be used to

5 generate redox power in the form of NADPH, e.g. through the
pentose phosphate pathway where sugar is converted to carbon
dioxide with parallel formation of NADPH, may be redirected
towards formation of citric acid resulting in a higher yield
of the product on the sugar. Similar reasoning will hold for

10 the production of other metabolites like succinic acid and
malic acid.

In the production of many amino acids, e.g. lysine by
Corynebacterium glutamicum, the amino acid is derived from

15 precursor metabolites of the central carbon metabolism.
Thus, in bacteria lysine is derived from oxaloacetate, which
again is derived from pyruvate or phosphoenolpyruvate. In
the conversion of sugar to the precursor metabolite there is
a net production of NADH and the overall conversion of sugar

20 to lysine therefore involves a net production of NADH. In
the conversion of oxaloacetate to lysine there is a net
consumption of NADPH (in some cases indirectly through the
use of glutamate which needs to be regenerated from 2-
oxoglutarate with expenditure of NADP). Expression of GAPN

25 may therefore lead to a reduced net formation of NADH and a
reduced net consumption of NADPH in the overall conversion of
sugar to lysine. Similar reasoning will hold for the
synthesis of other amino acids, e.g. isoleucine, threonine
and phenylalanine.

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In the production of many antibiotics, e.g. penicillin by
Penicillium chrysogenum, there is also a net production of

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NADH and a net consumption of NADPH in the overall conversion
of sugar to antibiotics. For these processes expression of
GAPN will therefore also be beneficial.

5   The enhanced expression of GAPN may be beneficial in
     combination with modulated (enhanced or suppressed)
     expression or activity of one or more other enzymes. Mention
     has been made above of interaction between GAPN expression
     and expression of lactate dehydrogenase in yeast. Generally,
10   the invention may be used to improve the metabolism of
     pentose sugars. The invention may be used to improve xylose
     uptake and to reduce xylitol secretion. In many
     microorganisms xylose metabolism involves xylose reductase
     (XR), which converts xylose to xylitol, xylitol dehydrogenase
15   (XDH), which converts xylitol to xylulose, and finally
     xylulose kinase that phosphorylates xylulose to xylulose-5-
     phosphate which enters the pentose phosphate pathway. XR
     involves formation of NADPH (the enzyme may use both NAD^+ and
     $NADP^+$ as co-factor, but it has preference for $NADP^+$) whereas
20   XDH involves consumption of NADH. There is therefore a net
     consumption of NADPH and a net formation of NADH upon xylose
     uptake. Thus, increased GAPN expression may, if it is
     linked with expression of xylose reductase, xylose
     dehydrogenase, and xylitol kinase, result in an increased
25   xylose uptake.

     Expression of GAPN or another said third enzyme may be
     provided for by the introduction into a micro-organism of one
     or more copies of a DNA coding sequence for the enzyme either
30   with an heterologous promoter or placed under the control of
     an native promoter sequence. Suitably, the coding sequence

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and an effective expression signal therefore is introduced in
a multi-copy plasmid.

In preferred embodiments, this invention specifically targets
5 the problem of production of surplus of NADH in functioning
cells and the problem with a limited supply of NADPH. The
cells are enabled to increase the formation of NADPH at the
cost of NADH formation. This is done for example, by
expression of a non-phosphorylating, $NADP^+$-dependent
10 glyceraldehyde-3-phosphate dehydrogenase (GAPN) (EC 1.2.1.9)
in a cell, as a means to alter the redox metabolism in the
cell.

GAPN catalyses the irreversible oxidation of glyceraldehyde-
15 3-phosphate and $NADP^+$ into 3-phosphoglycerate and NADPH. In
most cells the conversion of glyceraldehyde-3-phosphate into
3-phosphoglycerate is only catalysed by the sequential action
of two enzymes, i.e. NAD^+-dependent glyceraldehyde-3-
phosphate dehydrogenase (GAPDH) (EC 1.2.1.12) and
20 phosphoglycerate kinase (PGK) (EC 2.7.2.3) by conversion of
NAD^+ and ADP into NADH and ATP (see figure 1). The net
stoichiometry of the routes converting glyceralde-3-P into 3-
P-glycerate are:

25   **GAPN:**   glyceraldehyde-3-phosphate + $NADP^+$ → 3-
phosphoglycerate + NADPH
        GAPDH + PGK: glyceraldehyde-3-phosphate + NAD^+ + ADP + P
= 3-phosphoglycerate + NADH + ATP

30 The reaction catalysed by GAPN thereby yields one NADPH
instead of one NADH and one ATP when comparing with the total
reaction catalysed by GAPDH and PGK.

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The conversion of glyceraldehyde-3-phosphate into 3-
phosphoglycerate is part of the glycolysis, which is the main
energy-yielding pathway, and the reaction is therefore always
5   active when a cell is growing on hexose or pentose containing
substrates.

By controlling the amount of gapN expressed in the microbial
cell control can be exercised over how large a part of the
10   conversion of glyceraldehyde-3-phosphate into 3-
phosphoglycerate should go through GAPN. Thereby the redox
metabolism can be manipulated by controlling the amount of
one NAPDH formed at the expense of one NADH and ATP.

15   The Examples below make reference to the accompanying
drawings in which:
Figure 1 shows the results obtained in Example 8 in respect
of the profiles of biomass, glucose and xylose concentrations
(The symbols for xylose are filled circles and hollow
20   diamonds and the two curves lie close overlapping one
another); and
Figure 2 shows the results obtained in Example 8 in respect
of the profiles of ethanol, xylitol, glycerol and acetate
concentrations. (Closed and open square symbols are used for
25   ethanol and for acetate. The lower closed and open square
defined curves are acetate. The upper closed and open square
defined curves are ethanol.)

To illustrate this in the Examples below, a nucleotide
30   sequence containing the non-phosphorylating, $NADP^+$-dependent
glyceraldehyde-3-phosphate dehydrogenase gene (gapN) from
Streptococcus mutans was expressed in S. cerevisiae on a

multicopy plasmid.   The resulting strain was characterised in
anaerobic batch cultivations on the hexose glucose - a
typical method for ethanol production (Example 3) and on
xylose (Example 8).   NADP$^+$-dependent glyceraldehyde-3-
5   phosphate dehydrogenase activity was determined in Example 5
in both the *gapN* strain of Example 1 and in a reference
strain carrying the empty plasmid.   Activity of GAPN could
only be measured in the *gapN* strain and the activity was
approximately 10% of that of the NAD$^+$-dependent
10   glyceraldehyde-3-phosphate dehydrogenase activity.

The growth rate of the *gapN* strain of Example 1 was not
affected by the expression of GAPN activity when comparing to
the strain containing the empty plasmid.   The *gapN* strain
15   produced 43% less glycerol and 3% more ethanol.   A much
greater increase in ethanol production is achieved in Example
8.

Glycerol is formed by *S. cerevisiae* during anaerobic growth
20   to maintain the cytosolic redox balance. Under anaerobic
conditions NADH, produced as a result of production of
biomass and organic acids, can only be oxidised to NAD$^+$ by
formation of glycerol, since respiration is not possible and
the formation of ethanol is a redox-neutral process.   The
25   formation of glycerol is therefore a redox problem, so by
introducing *gapN* into *S. cerevisiae* the production of
glycerol will be reduced by one molecule for each molecule of
glyceraldehyde-3-phosphate that is converted via GAPN.   By
having a flux through GAPN that is high enough the production
30   of glycerol can be completely eliminated and the flux
redirected to ethanol and/or biomass thereby increasing the
ethanol yield.

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By increasing the amount of NADPH for biosynthesis by expressing *gapN* in a cell it can potentially be possible to increase the production of a product such as protein where

5   the supply of NADPH might be limiting.

Advantages of the exemplified approach include:

- Larger changes in yields can be achieved.
- Only one genetic change is required.
10   - The activity of the enzyme only affects the specific reaction it catalysed and the redox metabolism – there are no effects on other parts of metabolism and therefore the growth rate is not affected.
- By-production of glycerol in production of ethanol with
15   yeast can be eliminated.

Example 1 Expression of GAPN in *S. cerevisiae*.

Strains: *S. cerevisiae* (M4054, S288C MATa *ura3 gap1*) was used for construction of a reference strain and GAPN strain. For

20   long-term maintenance plasmid baring strains were grown to stationary phase in shake flask cultures on minimal media (see below). After addition of sterile glycerol to a concentration of 20% (vol/vol), aliquots were stored at -80°C. These frozen stocks were used for obtaining single

25   colonies on plates with a minimal medium (Verduyn et al., 1990), which were stored at 4°C, and used within 2 weeks for inoculation of precultures.

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Construction of reference strain: The empty pYX212 2μ high-copy vector containing the *URA3* gene and the *TPI1* promoter was transformed into *S. cerevisiae* (M4054) by electroporation.

5   **Construction of GAPN strain**: *gapN* was expressed on a pYX212 2μ high-copy vector containing the *URA3* gene and the *TPI1* promoter. The plasmid was constructed directly in *S. cerevisiae* (M4054) by cotransformation and homologous recombination between *Eco*RI digested pYX212 and PCR-amplified

10   *gapN* from *Streptococcus mutans*. PCR was performed on genomic DNA from *Streptococcus mutans* using Expand High Fidelity (Roche) and one primer identical to the *TPI1*promoter in pYX212 plus the first part of *gapN* (gapN-START-EcoRI-TPI promoter 5'-CTA CAA AAA ACA CAT ACA GGA ATT CAT GAC AAA ACA

15   ATA TAA AAA TTA TG) and a second primer (gapN-STOP-NcoI-BamHI-AvrII-ApaI 5'-GGG CCC TAG GAT CCA TGG TGA ATT TTA TTA TTT GAT ATC AAA TAC GAC GG) identical to the MCS of pYX212 and the last part of *gapN* including the stop codon. Hence the ORF of *gapN* has been cloned between *Eco*RI and *Nco*I side in

20   pYX212, down stream the *TPI1* promoter. The original start codon TTG was substituted with an ATG, to make translation in *S. cerevisiae* possible. Construction was verified by diagnostic PCR.

Example 2 Shake flask cultivations and precultures: Aerobic
shake flask cultivations were performed in baffled, cotton-
stopped, 500 ml Erlenmeyer flasks to screen transformants

5   obtained. Precultures for anaerobic batch cultivations were
grown I similar flasks but without baffles. These flasks
contained each 100 ml of a defined mineral medium containing
7.5 g/L $(NH_4)_2SO_4$; 14 g/L KH_2PO_4; 0.5 g/L $MgSO_4$, $7H_2O$; 50 µl/L
antifoam (Sigma A-8436); 2% (w/vol) glucose; trace metals (15

10   mg/L EDTA; 4.5 mg/L $ZnSO_4$, $7H_2O$; 0.84 mg/L $MnCl_2$, $2H_2O$; 0.30
mg/L $CoCl_2$, $6H_2O$; 0.30 mg/L $CuSO_4$, H_2O; 0.40 mg/L Na_2MoO_4,
$2H_2O$; 4.5 mg/L $CaCl_2$, $2H_2O$; 3.0 mg/L $FeSO_4$, $7H_2O$; 1.0 mg/L
H_3BO_3; and 0.10 mg/L KI) and vitamins (0.05 mg/L D(-)biotin;
1.0 mg/L Ca D(+)panthotenate; 1.0 mg/L nicotinic acid; 25

15   mg/L myo-inositol; 1.0 mg/L thiamine chloride hydrochloride;
1.0 mg/L pyridoxol hydrochloride; and 0.20 mg/L p-
aminobenzoic acid). The pH of the mineral medium was set to
6.5 with NaOH and autoclaved separately from the glucose
solution. After autoclavation the vitamin solution was added

20   to the flasks by sterile filtration. Shake flasks and
precultures were inoculated with a single colony from plate
cultures a grown at 30°C and 150 rpm. Precultures were grown
to exponential phase and used for inoculation of anaerobic
batch cultivations to a start concentration of 1 mg CDW/L.

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Growth of both the reference and the GAPN-strain was
observed.

Example 3 Anaerobic batch cultivations: Cultivations were

5   carried out in well-controlled laboratory fermentors (B.
Braun Biotech, Germany) with a working volume of 2 litres. A
defined mineral medium (Verduyn et al., 1990) was used, which
contained per litre: 40 g glucose; 5.0 g $(NH_4)_2SO_4$; 3.0 g
KH_2PO_4; 0.5 g $MgSO_4$, $7H_2O$; and trace metals and vitamins as

10   described in shake flask cultivations and precultures. 300
$\mu l/L$ antifoam (Sigma A-8436) was added to avoid foaming and
the medium was supplemented with 420 mg/L Tween 80 and 10
mg/L ergosterol, which is necessary for anaerobic growth of
S. cerevisiae. The glucose solution was autoclaved separately

15   from the mineral medium and afterwards added to the fermentor
together with a sterile filtrated solution containing the
vitamins and together with the Tween 80 and ergosterol, which
first were dissolved in boiling pure ethanol.
Cultivations were carried out at 30°C with a stirrer speed of

20   600 rpm and were flushed with nitrogen gas at a flow rate of
400 ml per minute. To minimise the diffusion of O_2 into the
cultures, the bioreactors were fitted with Norprene tubing.
The concentration of dissolved oxygen was measured with
Mettler Toledo polarographic electrode and remained below the

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detection limit. pH was kept at 5.0 by automatic addition of
4 M KOH. The bioreactors were fitted with cooled condensers,
and the off-gas was led to a gas analyser (INNOVA, Denmark)
to measure the content of CO_2. The biomass concentration

5   during fermentation with the reference and GAPN strains are
shown in Figure 2. It is found that the maximum specific
growth rate of the GAPN strain is identical with that of the
reference strain.

10   **Example 4 Analysis of extracellular metabolites:** Culture
samples for determination of glucose, ethanol, glycerol,
acetate, pyruvate and succinate concentrations were filtered
through a 0.45 µm cellulose acetate filter (Osmonics)
immediately after sampling, and the filtrate was frozen at -

15   20°C until further analysis. The concentrations of the
metabolites were determined by high-pressure liquid
chromatography on an Aminex HPX-87Hm column (Bio-Rad) kept at
65°C and eluted at 0.6 ml per minute with 5 mM H_2SO_4. Acetate
and pyruvate were detected spectrophotometrically by a Waters

20   486 Turnable Absobance Detector at 210 nm. Glucose, ethanol,
glycerol and succinate were detected refractometrically by a
Waters 410 Differential Refractometer. Measurement of the
metabolites during anaerobic fermentations is shown in Figure

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3. The final concentrations of the metabolites are listed
below. It is seen that the GAPN strain produces more ethanol
and less glycerol.

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	Ethanol (g/L)	Glycerol (g/L)	Pyruvate (g/L)
Reference strain	14,90	2,81	0,061
GAPN strain	17,05	2,06	0,066

Based on the measurements of glucose the overall conversion
yields to the different metabolites were calculated and they
10 are given below.

	Ethanol (g/g glc)	Glycerol (g/g glc)	Pyruvate (g/g glc)
Reference strain	0,392	0,078	0,0013
GAPN strain	0,403	0,0463	0,0012

It is observed that the ethanol yield is improved 3% and the
glycerol yield is reduced by 40%.

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Example 5 Measurement of enzyme activities: Cell free

extracts were produced with the help of a Fastprep FP120

instrument (Savant Instruments, New York) as described by

Møller et al. (2002).

5   Enzyme activities were assayed at 30°C by following the NADPH

or NADH production at 340 nm using a spectrophotometer (HP

8353 UV-VIS system with Chemstation software from Hewlett

Packard). Glyceraldehyde-3-phosphate dehydrogenase activity

was determined as described by Crow and Wittenberger (1979)

10   in a 1 ml reaction mixture activity. For determination of

non-phosphorylating, $NADP^+$-dependent glyceraldehyde-3-

phosphate dehydrogenase activity the reaction mixture

contained: 125 mM triethanolamine/HCl buffer (pH 8.3), 1 mM

$NADP^+$, 5 mM 2-mercaptoethanol, and cell free extract. NAD^+-

15   dependent glyceraldehyde-3-phosphate dehydrogenase activity

was determined with a reaction mixture containing: 125 mM

triethanolamine/HCl buffer (pH 8.3), 1 mM NAD^+, 5 mM

cystein/HCl, and cell free extract. The reactions were

started by adding DL-glyceraldehyde-3-phosphate (prepared

20   from DL-glyceraldehyde-3-phosphate diethyl acetal, Sigma G-

5376) to a final concentration of 2 mM. Protein content in

cell free extracts was determined by the Lowry method, using

fatty-acid free BSA (Sigma A-6003) as standard. Results from

analysis of the enzyme activity are given below. It is seen

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that in the reference strain there is no activity of a NADP-
dependent glyceraldehydes dehydrogenase whereas in the GAPN
strain there is some low activity (accounting for about 10%
of the total glyceraldehydes dehydrogenase activity).

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	Reference strain	GAPN strain
NAD-dependent glyceraldehyde dehydrogenase	11,2 ± 0,74	7,03 ± 0,71
NADP-dependent glyceraldehyde dehydrogenase	0	0,65 ± 0,03

Example 6 Sequencing of GapN

10   In order to verify that no mutations had occurred in the gapN

encoding gene expressed in the 2μ high copy number plasmid,

the part of the plasmid containing the gapN was sequenced.

The sequence of the gapN as inserted into the plasmid and as

found actually to be present therein was 'atg aca aaa caa tat

15   aaa aat tat gtc aat ggc gag tgg aag ctt tca gaa aat gaa att

aaa atc tac gaa ccg gcc agt gga gct gaa ttg ggt tca gtt cca

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    gca atg agt act gaa gaa gta gat tat gtt tat gct tca gcc aag

    aaa gct caa cca gct tgg cga tca ctt tca tac ata gaa cgt gct

    gcc tac ctt cac aag gta gca gat att ttg atg cgt gat aaa gaa

    aaa ata ggt gct gtt ctt tcc aaa gag gtt gct aaa ggt tat aaa

 5  tca gca gtc agc gaa gtt gtt cgt act gca gaa atc att aat tat

    gca gct gaa gaa ggt ctt cgt atg gaa ggt gaa gtc ctt gaa ggc

    ggc agt ttt gaa gca gcc agc aag aaa aaa att gcc gtt gtt cgt

    cgt gaa cca gta ggt ctt gta tta gct att tca cca ttt aac tac

    cct gtt aac ttg gca ggt tcg aaa att gca ccg gct ctt att gcg

10  gga aat gtt att gct ttt aaa cca ccg acg caa gga tca atc tca

    ggg ctc tta ctt gct gaa gca ttt gct gaa gct gga ctt cct gca

    ggt gtc ttt aat acc att aca ggt cgt ggt tct gaa att gga gac

    tat att gta gaa cat caa gcc gtt aac ttt atc aat ttt act ggt

    tca aca gga att ggg gaa cgt att ggc aaa atg gct ggt atg cgt

15  ccg att atg ctt gaa ctc ggt gga aaa gat tca gcc atc gtt ctt

    gaa gat gca gac ctt gaa ttg act gct aaa aat att att gca ggt

    gct ttt ggt tat tca ggt caa cgc tgt aca gca gtt aaa cgt gtt

    ctt gtg atg gaa agt gtt gct gat gaa ctg gtc gaa aaa atc cgt

    gaa aaa gtt ctt gca tta aca att ggt aat cca gaa gac gat gca

20  gat att aca ccg ttg att gat aca aaa tca gct gat tat gta gaa

    ggt ctt att aat gat gcc aat gat aaa gga gcc act gcc ctt act

    gaa atc aaa cgt gaa ggt aat ctt atc tgt cca atc ctc ttt gat

    aag gta acg aca gat atg cgt ctt gct tgg gaa gaa cca ttt ggt

    cct gtt ctt ccg atc att cgt gtg aca tct gta gaa gaa gcc att
```

22

gaa att tct aac aaa tcg gaa tat gga ctt cag gct tct atc ttt

aca aat gat ttc cca cgc gct ttt ggt att gct gag cag ctt gaa

gtt ggt aca gtt cat atc aat aat aag aca cag cgc ggc acg gac

aac ttc cca ttc tta ggg gct aaa aaa tca ggt gca ggt att caa

5   ggg gta aaa tat tct att gaa gct atg aca act gtt aaa tcc gtc

gta ttt gat atc aaa', which is identical with the sequence of

gapN from *Streptococcus mutans*.

Example 7 Expression of GAPN in a xylose metabolising strain

10   **of *S. cerevisiae***

Strains: *S. cerevisiae* (*MATa SUC2 MAL2-8 pADH-XYL1 pPGK-XYL2*

pPGK-XKS1 ura3⁻) was used for construction of a xylose

metabolising strain of *S. cerevisiae* expressing GAPN. For

long-term maintenance, plasmid bearing strains were grown to

15   stationary phase in shake flask cultures on minimal media

(see below). After addition of sterile glycerol to a

concentration of 20% (vol/vol), aliquots were stored at –

80°C. These frozen stocks were used for obtaining single

colonies on plates with a minimal medium (Verduyn et al.,

20   1990), which were stored at 4°C, and used within 2 weeks for

inoculation of precultures.

Construction of reference strain: The empty pYX212 2μ high-

copy vector containing the *URA3* gene and the *TPI1* promoter

23

was transformed into *S. cerevisiae* (*MATa SUC2 MAL2-8 pADH-XYL1 pPGK-XYL2 pPGK-XKS1 ura3⁻*) by the LiAc method and positive clones, containing the pYX212 *URA3* 2µ plasmid, were selected on synthetic minimal ammonium medium with glucose.

5 **Construction of GAPN strain**: The pYX212 2µ high-copy vector containing the *URA3* gene and the *TPI1* promoter in front of the *gapN* encoding gene was transformed into *S. cerevisiae* (*MATa SUC2 MAL2-8 pADH-XYL1 pPGK-XYL2 pPGK-XKS1 ura3⁻*) by the LiAc method, and positive clones, containing *gapN* down stream

10 the *TPI1* promoter in the *URA3* 2µ plasmid, were selected on synthetic minimal ammonium medium with glucose.
A number of transformants was tested in aerobic shake flasks on glucose for growth rate and GAPN activity. A transformant was chosen with unchanged growth rate and high GAPN activity

15 and denoted Xyl GAPN strain - the reference strain was denoted Xyl reference strain. Both strains had similar xylose reductase and xylose dehydrogenase activities, which were within the expected range.

20 **Example 8 Anaerobic batch cultivations of a xylose metabolising strain of *Saccharomyces cerevisiae***: Cultivations were carried out in well-controlled laboratory fermentors with a working volume of 4 litres. A defined mineral medium

24

(Verduyn et al., 1990) was used, which contained per litre: 20 g glucose; 50 g xylose; 5.0 g $(NH_4)_2SO_4$; 3.0 g KH_2PO_4; 0.5 g $MgSO_4$, $7H_2O$; and trace metals and vitamins as described in shake flask cultivations and precultures. 500 µl/L antifoam

5   (Sigma A-8436) was added to avoid foaming and the medium was supplemented with 420 mg/L Tween 80 and 10 mg/L ergosterol, which is necessary for anaerobic growth of *S. cerevisiae*. The glucose solution was autoclaved separately from the mineral medium and afterwards added to the fermentor together with a

10   sterile filtered solution containing the vitamins and together with the Tween 80 and ergosterol, which first were dissolved in boiling pure ethanol.

Cultivations were carried out at 30°C with a stirrer speed of 500 rpm and were flushed with nitrogen gas at a flow rate of

15   800 ml per minute. To minimise the diffusion of O_2 into the cultures, the bioreactors were fitted with Norprene tubing. pH was kept at 5.0 by automatic addition of 2 M NaOH. The bioreactors were fitted with cooled condensers, and the off-gas was led to a gas analyser (INNOVA, Denmark) to measure

20   the content of CO_2. The profiles of the concentrations of biomass, glucose and xylose for both the Xyl reference strain (closed symbols) and the Xyl GAPN strain (open symbols) are shown in Figure 1. The profiles of the concentrations of ethanol, xylitol, glycerol and acetate for both the Xyl

reference strain (closed symbols) and the Xyl GAPN strain

(open symbols) are shown in Figure 2. It is found that the

glucose and xylose uptake is approximately the same in the

two strains whereas the Xyl GAPN strain produces much more

5   ethanol, much less xylitol and less glycerol compared with

the Xyl reference strain. The Xyl GAPN strain produces

slightly more acetate than the Xyl reference strain. After 50

hours of fermentation the following concentrations of the

metabolic products were obtained:

10

	Ethanol (g/L)	Glycerol (g/L)	Xylitol (g/L)
Xyl reference strain	12.3	3.5	13.4
Xyl GAPN strain	14.1	1.9	7.2

This corresponds to a 15% increase in the ethanol

concentration, a 45% decrease in glycerol concentration and a

45% decrease in xylitol concentration.

15

During growth on glucose there is a slightly higher

production of ethanol and a smaller production of glycerol by

the Xyl GAPN strain (in analogy with results of Example 4).

During growth on xylose overall the yield coefficients were

20   estimated and are given below.

	Ethanol (g/g sugar)	Glycerol (g/g sugar)	Xylitol (g/g sugar)
Xyl reference strain	0.22	0.065	0.33
Xyl GAPN strain	0.29	0.037	0.17

These yield coefficients corresponds approximately with a 30%
increase in the ethanol yield, a 40% decrease in glycerol

5   yield and an almost 50% decrease in xylitol yield.

Example 9 Anaerobic chemostat cultivations of a xylose

metabolising strain of Saccharomyces cerevisiae

10 Anaerobic steady-state chemostat cultures were obtained in

well-controlled 2-litre jacketed bioreactors (B. Braun

Biotech, Melsungen, Germany) with a constant working volume

of 1.0 litre and a dilution rate of 0.05 h^{-1} (which equals

the specific growth rate). Cultivations were carried out at

15 30°C with a stirrer speed of 500 rpm and were flushed with

pure nitrogen gas (0.2 liter·min^{-1}). pH was kept at 5.0 by

automatic addition of 2 M NaOH. To minimise the diffusion of

O_2 into the cultures, the bioreactors were fitted with

Norprene tubing. The concentration of dissolved oxygen was

20 measured with Mettler Toledo polarographic electrode and

remained below the detection limit. The bioreactors were
fitted with cooled condensers (5°C), and the off-gas was led
to a gas analyser (INNOVA, Denmark) to measure the content of
CO_2. Steady state was assumed when at least five residence

5 times had passed since starting the continuous cultivation
and was verified by constant (±2%) biomass concentration,
metabolite concentrations, and carbon dioxide evolution rate
at two measurements taken over 2 residence times. The
cultures were fed with a defined medium similar to that

10 described in Example 8.

Precultures for chemostat cultivations were grown at 30°C and
150 rpm for 16-24 hours in cotton-stopped, 500 ml Erlenmeyer
flasks with baffles containing each 100 ml of a media of pH
6.5 similar to that in the fermenters, except from different

15 concentrations of glucose (20 $g \cdot l^{-1}$), $(NH_4)_2SO_4$ (7.5 $g \cdot l^{-1}$),
KH_2PO_4 (14 $g \cdot l^{-1}$), and antifoam (50 $\mu l \cdot l^{-1}$), and furthermore, no
Tween or ergosterol were added. Precultures were inoculated
from plate cultures grown at 30°C. Continuous cultures were
inoculated with 20 ml exponential growing preculture.

20

In the chemostat culture at steady state the concentrations
of ethanol, glycerol and xylitol were measured, and the
results are given below.

	Ethanol (g/L)	Glycerol (g/L)	Xylitol (g/L)
Xyl reference strain	10.84	1.49	7.41
Xyl GAPN strain	12.05	0.55	4.53

Based on the measurements of sugars the overall conversion yields to the different metabolites were calculated and they are given below.

	Ethanol (g/g sugar)	Glycerol (g/g sugar)	Xylitol (g/g sugar)
Xyl reference strain	0.29	0.044	0.21
Xyl GAPN strain	0.36	0.018	0.14

It is observed that the ethanol yield is improved by 24% and the glycerol yield is reduced by almost 60% and that the xylitol yield is reduced by 33%.

In this specification, unless expressly otherwise indicated, the word 'or' is used in the sense of an operator that returns a true value when either or both of the stated conditions is met, as opposed to the operator 'exclusive or' which requires that only one of the conditions is met. The

word 'comprising' is used in the sense of 'including' rather than in to mean 'consisting of'.

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